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Phosphoglucose Isomerase Mutant of *Rhizobium meliloti*

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A mutant strain of complex phenotype was selected in *Rhizobium meliloti* after nitrosoguanidine mutagenesis. It failed to grow on mannitol, sorbitol, fructose, mannose, ribose, arabitol, or xylose, but grew on glucose, maltose, gluconate, L-arabinose, and many other carbohydrates. Assay showed the enzyme lesion to be in phosphoglucose isomerase (*pgi*), and revertants, which were of normal growth phenotype, contained the enzyme again. Nonpermissive substrates such as fructose and xylose prevented growth on permissive ones such as L-arabinose, and in such situations there was high accumulation of fructose 6-phosphate. The mutant strain had about 20% as much exopolysaccharide as the parent. Nitrogen fixation by whole plants was low and delayed when the mutant strain was the inoculant.

Carbohydrate metabolism in *Rhizobium* has not been extensively studied. Radiorespirometric and enzymological experiments suggest that the Entner-Doudoroff pathway may be a principal route of sugar degradation (12, 13, 17, 18). Some enzymes of the pentose-phosphate pathway and of glycolysis are also present (17, 19), but the glycolytic pathway seems to be incomplete in many strains (17). There is some knowledge of specific degradative pathways: e.g., mannitol and arabitol (15), sucrose and fructose (16, 15), glycerol (2), and arabinose (5, 21).

We have now selected a pleiotropic mutant of *R. meliloti* affected in growth on many carbohydrates. The mutant lacks phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9), and its characteristics serve to clarify the possible pathways of carbohydrate metabolism.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The parent strain employed was *R. meliloti* L5-30 (*arg str*^{r}), which was kindly supplied by J. Dénarié, Station de Pathologie Végétale, CNRA, Versailles, France. The parent and mutant strains were kept at 4°C on Nutman's medium (20) with glycerol (1%) substituted for mannitol and were transferred at monthly intervals.

The cells were grown aerobically at 30°C on a minimal medium (23) containing KNO_3 (0.6 mg/ml), and it was always supplemented with L-arginine (25 μg/ml), biotin (0.2 μg/ml), streptomycin sulfate (0.1 mg/ml), and a carbon source (5 mg/ml). The final pH was 7.2. Plates also contained 2% agar. All sugars, with the exception of L-arabinose, were of the D configuration.

Growth rates were determined by turbidimetric measurements at 660 nm in side-arm flasks with a Coleman spectrophotometer. An absorbance value of unity was found to correspond to 1.6 mg of wet cells. Viable-cell counts were made on minimal medium containing 2% agar and 1% sugar.

Isolation of mutant and revertant. Strain L5-30, 10^9 cells/ml in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 6.5), was treated with 200 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 30°C for 30 min (1). The treated cells (40% survival) were washed in the same buffer and grown out in minimal medium with glycerol (0.5%) for 24 h. A subculture was inoculated to mannitol minimal medium, and penicillin G (2,000 U/ml) was added in early logarithmic phase. After 20 h, the surviving cells (1%) were cloned on glycerol minimal plates. After 7 days, ca. 2,000 colonies were patched on mannitol minimal medium. Four mannitol-negative strains were obtained; one, strain UR1, was used for further studies. The spontaneous revertant strain UR2 was obtained by spreading a dense culture on minimal medium with fructose as sole carbon source.

Cell-free extract preparation. Cells were harvested at the beginning of the stationary phase by use of a Sorvall centrifuge at 11,600 × *g* for 30 min and were washed twice in 0.005 M sodium phosphate buffer (pH 7.0). The cell pellet was suspended in an equal volume of 0.005 M sodium phosphate buffer (pH 7.0), disrupted by a French press at 16,000 lb/in^2 (three 1-min treatments), and centrifuged at 16,000 × *g* for 30 min. The supernatant fluid was employed for the measurement of the enzyme activities. All the operations were carried out between 0 and 4°C.

Enzyme assays. Phosphoglucose isomerase was measured by following the reduction of nicotinamide adenine dinucleotide phosphate (NADP) at 340 nm in a Beckman spectrophotometer model DU. The reaction mixture contained (in a total volume of 1.0 ml): sodium fructose 6-phosphate, 2 μmol; Tris buffer (pH

7.6), 100 μmol; glucose 6-phosphate dehydrogenase, 1 μg (Sigma Chemical Co.); NADP, 0.5 μmol; and cell-free extract.

NADP-linked glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and NADP-linked gluconate 6-phosphate dehydrogenase (EC 1.1.1.43) were determined by following the formation of reduced NADP. The incubation mixture contained (1.0 ml): sodium glucose 6-phosphate, 2 μmol, or sodium 6-phosphogluconate, 2.5 μmol; glycylglycine buffer (pH 8.0), 80 μmol; NADP, 0.3 μmol; $MgSO_4$, 10 μmol; and cell-free extract.

Phosphofructokinase (EC 2.7.1.11) was determined by the method of Ling et al. (14). Glucokinase (EC 2.7.1.2) was determined by the method of Fraenkel and Horecker (9). Phosphogluconate dehydratase (EC 4.2.1.12), the first enzyme of the Entner-Doudoroff pathway, was assayed by a slight modification of the method of Fradkin and Fraenkel (6). The incubation mixture (1.3 ml) contained: Tris buffer (pH 7.6), 80 μmol; sodium 6-phosphogluconate, 8 μmol; $MgCl_2$, 10 μmol; reduced glutathione, 2.4 μmol; 0.68 mg of protein of an extract of *Escherichia coli* strain DF1070 (*gnd edd eda*$^+$) (8) to supply 2-keto-3-deoxy-6-phosphogluconate aldolase in excess; and cell-free extract. At 0, 10, 20, and 30 min of incubation, 0.1-ml samples were added to 0.1 ml of 2,4-dinitrophenylhydrazine (1 mg/ml in 2 N HCl), and 5 min later 5 ml of 2.5 N NaOH was added. The optical density was read at 430 nm, and sodium pyruvate was used as standard.

2-Keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) was determined by the method of Fradkin and Fraenkel (6). Protein was measured by the method of Bücher (4), which was standardized with bovine serum albumin.

Determination of fructose 6-phosphate. The acid-soluble pool was prepared by a modification of the method of Böck and Neidhardt (3). The cells from 40 ml of chilled culture were collected by centrifugation, the culture medium was separated, and the precipitate was suspended in 0.2 ml of 10% $HClO_4$ at 0°C. After 30 min of incubation at 0°C, the mixture was neutralized with 2 N KOH and clarified by centrifugation; the precipitate was discarded. Fructose 6-phosphate assay mixture (1 ml) contained: Tris-hydrochloride (pH 7.6), 100 μmol; $MgSO_4$, 10 μmol; NADP, 0.2 μmol; glucose 6-phosphate dehydrogenase (Sigma), 1 μg; phosphoglucose isomerase (Sigma), 1 μg; and sample. Fructose 6-phosphate was calculated according to the molar absorption of NADPH, $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Extracellular polysaccharide production. Cells were grown in minimal medium containing 1% arabinose, and samples were taken after different periods of time. Extracellular polysaccharide was prepared as follows. The cultures were centrifuged to remove the bacteria, and the supernatant fluids were dialyzed at 4°C for 48 h against repeated changes of distilled water. The polysaccharide was then precipitated from the solution by adding 3 volumes of ethanol. After standing at 4°C overnight, the polysaccharide was collected by centrifuging and was dissolved in distilled water. Polysaccharide yields were determined by the anthrone method with a glucose standard (11).

Nitrogen fixation assays. Nitrogen fixation activity in root nodules induced by strains L5-30, UR1, and UR2 was followed by application of an acetylene reduction assay (10). Plants of lucerne (*Medicago sativa* var. Du Puits) were grown on 20 ml of N-free nutrient agar enclosed in test tubes (200 by 25 mm) stoppered with cotton wool and maintained at 22°C in a light-controlled room with a photoperiod of 14 h. The seedlings were inoculated 10 days after sowing with 1 ml of the proper rhizobium culture grown up to 10^8 cells per ml. Two kinds of assays were performed to evaluate the $N_2(C_2H_2)$-fixing activity. In type A assays, the shoots of the plants were discarded, and the nodulated roots, freed from the agar and surface-dried, were kept in 25-ml rubber-stopped bottles. Acetylene gas was injected into the vessels to a final concentration of 10%, and, after an incubation period of 2 h at 25°C, 2-ml gas samples were withdrawn for the measurement of ethylene content. In type B assays the test tubes with whole plants were stoppered with rubber caps, and acetylene was added to a concentration of 10% by volume. Gas samples (2 ml) were taken after 10 h of incubation at 25°C. All assays were run with plants at 4 h of the light period.

Ethylene formation was followed by gas chromatography with the use of a column (1.5 m by 3 mm) packed with Porapak Q (80/100 mesh) at 20°C and a flame ionization detector on a Varian 1860 gas chromatograph. Ethylene peaks were identified and measured by introducing into the gas chromatograph a known volume of an ethylene standard.

RESULTS

A mutant selection was performed for strains unable to grow on mannitol but still able to grow on glycerol (see Materials and Methods). Mutant strain UR1 proved to have a complex growth phenotype (Table 1). It showed no growth on mannitol, fructose, sorbitol, mannose, xylose, ribose, or arabitol. It did grow on many other carbon sources, including glucose, glycerol, L-arabinose, pyruvate, and succinate. On some of these, such as L-arabinose, growth seemed normal, whereas on others, such as sucrose, growth was much slower than that of the parental strain. Spontaneous revertants were selected on several of the nonpermissive carbon sources. Strain UR2, obtained on fructose, grew like the wild type again.

Table 2 shows assays of several enzymes of intermediary metabolism. The phosphoglucose isomerase activity of the mutant was about 3% of normal, and revertant strain UR2 had regained the activity. Table 3 shows that the phosphoglucose isomerase level in the parent varied little when grown on several different carbon sources; the mutant level was low in all cases, and the level in the revertant strain was also constitutive (but much higher than that of the wild type).

Growth was also tested on a number of combinations of carbon sources. Many of the nonpermissive carbon sources prevented growth on

TABLE 1. *Growth of parent (L5-30) and mutant (UR1) strains on several carbon sources*

Carbon source	Colony size[a]	
	L5-30	UR1
Fructose	2.5 (6)[b]	0.0
Mannitol	3.0 (8)	0.0
Ribose	2.3 (9)	0.0
Sorbitol	1.9 (7)	0.0
Mannose	1.4 (6.5)	0.0
Arabitol	2.7 (4)	0.0
Xylose	1.9 (9)	0.0
Arabinose	1.4 (8.5)	1.5 (8.5)
Succinate	1.6 (8)	1.0 (16)
Pyruvate	1.4 (ND)	1.5 (ND)
Lactose	1.0 (ND)	0.8 (ND)
Maltose	1.6 (ND)	1.2 (ND)
Glucose	1.5 (7.5)	1.0 (8.5)
Glycerol	1.6 (10.5)	1.0 (16)
Sucrose	1.4 (6.5)	0.6 (18)
Gluconate	0.8 (ND)	0.6(ND)

[a] Cultures were spread on minimal medium plates so as to give ca. 50 colonies per plate. After 8 days of incubation at 30°C, average colony sizes were estimated and are reported in millimeters.

[b] Doubling times (hours) in liquid minimal medium. ND, Not determined.

TABLE 2. *Enzymes of glucose metabolism in cells of the parent (L5-30), mutant (UR1), and revertant (UR2) strains grown on glucose*[a]

Enzyme[b]	Specific activity[c]		
	L5-30	UR1	UR2
Glucokinase	87	65	95
Glucose 6-phosphate dehydrogenase	184	163	192
Gluconate 6-phosphate dehydrogenase	52	75	60
Phosphoglucose isomerase	560	17	4,950
Phosphofructokinase	0.5	0.5	0.5
Phosphogluconate dehydratase	16	45	ND[d]
2-Keto-3-deoxy-6-phosphogluconate aldolase	125	118	ND

[a] Cell-free extracts were prepared as described in the text.

[b] For enzyme assays, see text.

[c] Expressed as nanomoles of NADP reduced or NADH oxidized per minute per milligram of protein, except in the case of phosphogluconate dehydratase activity, which is expressed as nanomoles of pyruvate formed per minute per milligram of protein.

[d] ND, Not determined.

permissive carbon sources. Thus, on the following minimal plates with two substrates, each 1%, the mutant formed no colonies: mannitol plus glycerol or gluconate; ribose plus succinate, glucose, or arabinose; and fructose plus glycerol, arabinose, gluconate, or succinate. It did grow on mannitol plus arabinose or succinate and on sorbitol plus arabinose. Figure 1 illustrates the situation in liquid medium for the mixture of glucose (permissive) and fructose (nonpermissive); in this condition inhibition lasted about 20 h.

The phenomenon of inhibition of growth by sugars whose metabolism is blocked is sometimes associated with accumulation of metabolic intermediates at the block (3, 7). Table 4 shows that fructose 6-phosphate was accumulated to very considerable levels in the mutant exposed to either fructose or xylose. Even with growth on the permissive medium, glucose, the fructose 6-phosphate level was higher than in the parental strain. Some fructose 6-phosphate also accumulated in the medium. The relief of growth inhibition, after prolonged incubation, was ac-

TABLE 3. *Phosphoglucose isomerase activity in parent (L5-30), mutant (UR1), and revertant (UR2) strains grown on different carbon sources*[a]

Carbon source	Specific activity[b]		
	L5-30	UR1	UR2
Glucose	560	17	4,950
Arabinose	505	16	3,870
Glycerol	420	5	3,650

[a] Enzyme assay and cell-free extract preparation are described in the text.

[b] Expressed as nanomoles of NADP reduced per minute per milligram of protein.

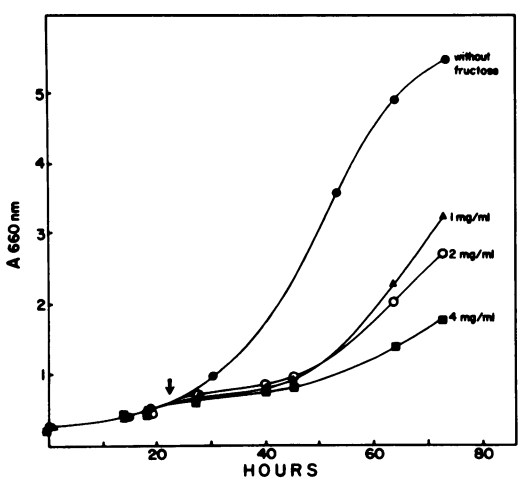

FIG. 1. *Effect of different fructose concentrations on the growth of mutant UR1 in glucose minimal medium. Growth was followed by turbidity measurements and plotted as a function of time for four parallel cultures. Fructose was added at the time indicated by the arrow to give a final concentration of 1 mg/ml (▲), 2 mg/ml (○), or 4 mg/ml (■), except for the culture which was run as a blank (●).*

companied by a fall in the internal level of fructose 6-phosphate.

Exopolysaccharide production in parent, mutant, and revertant strains is shown in Table 5. In comparison with the parent, the amount of extracellular polysaccharide found in the culture media in which the mutant cells had grown was about one-fifth as large.

The mutant was somewhat defective in nodulation (Table 6). As tested 3 weeks after inoculation by assay A (using the roots alone), formation of ethylene by the mutant strain was not above the blank value. A whole-plant assay (assay B) gave similar results: substantial ethylene reduction in parent and revertant strain and marginal fixation by the mutant. With 60-day-old plants, however, the ethylene reduction test showed the mutant to have about one-fourth the capacity of the parental strain. The nodules at this time still contained the mutant, as no colonies with revertant phenotype could be obtained after many isolations were performed.

DISCUSSION

The experiments described in this paper, together with previous studies, are in accord with the general pattern of carbohydrate metabolism in *R. meliloti* shown in Fig. 2. Thus, the mutant, being blocked at phosphoglucose isomerase and having no phosphofructokinase, fails to grow on mannitol, mannose, sorbitol, and fructose. Growth occurs on substances which enter metabolism past the block: e.g., glucose, gluconate, glycerol, pyruvate, etc. The metabolism of ribose, xylose, and arabitol may be by the nonoxidative pentose-phosphate pathway. The major product of that pathway being fructose 6-phosphate, there would be no growth on those pentoses. L-Arabinose, on the other hand, is used by a nonphosphorylated pathway leading to α-ke-

TABLE 5. *Extracellular polysaccharide production*[a]

Strain	Polysaccharide[b]
L5-30 (parent)	21.5
UR1 (mutant)	3.9
UR2 (revertant)	19.8

[a] Results shown are for cultures after 6 days of growth (stationary phase) in minimal medium containing 1% arabinose.

[b] Anthrone determination, calculated as micrograms of glucose per 10^{10} viable cells.

TABLE 6. *Specific activities of N_2 [C_2H_2] fixation by lucerne root nodules induced by parent (L5-30), mutant (UR1), and revertant (UR2) strains*[a]

Expt	Fixation (nmol/h per plant)[b]			
	L5-30	UR1	UR2	Blank[c]
Assay A, at 21 days	17.1	6.8	22.0	9.2
Assay B, at 21 days	35.8	6.1	32.0	5.5
Assay B, at 60 days	125.6	40.2	ND[d]	6.7

[a] Fixation was measured on roots (assay A) or whole plants (assay B) 21 or 60 days after inoculation, with the use of lucerne (*Medicago sativa* var. DuPuits) as described in Materials and Methods.

[b] The data shown are mean values obtained from two different plants.

[c] Uninoculated control.

[d] ND, Not determined.

TABLE 4. *Fructose 6-phosphate accumulation*[a]

Strain	Carbon source	Inhibitor added	Time after inhibitor addition (h)	Fructose 6-phosphate	
				Inside (nmol/g)	Outside (nmol/ml)
L5-30 (parent)	Glucose			160	4
	Fructose			420	(0)
	Xylose			530	(2)
UR1 (mutant)	Glucose			900	(2)
	Glucose	Fructose	7	3,080	ND[b]
	Glucose	Fructose	10	2,930	25
	Glucose	Fructose	12	3,250	40
	Glucose	Fructose	36	1,300	70
	Glucose	Xylose	7	4,870	14
	Glucose	Xylose	12	4,920	24
	Glucose	Xylose	36	1,660	44

[a] Cultures were in logarithmic growth in minimal medium containing 5 mg of the specified carbon source per ml when fructose or xylose was added to a final concentration of 2 mg/ml. Fructose 6-phosphate was determined in cells and supernatant by an enzymatic method (see text). Values in parentheses are below the limit of resolution of the assay method. Fructose 6-phosphate internally was expressed as nanomoles per gram of wet cells and outside the cells as nanomoles per milliliter of medium. Cell density at time of addition of inhibitor was 1.6 mg (wet weight)/ml.

[b] ND, Not determined.

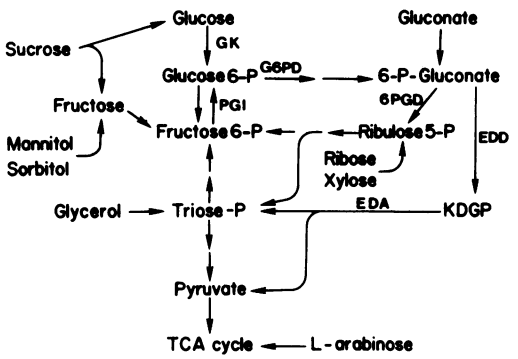

FIG. 2. *Outline of possible pathways on carbohydrate metabolism in R. meliloti. Pathway intermediates and enzymes are abbreviated as follows: 6-phosphogluconate dehydrogenase (6PGD), glucose 6-phosphate dehydrogenase (G6PD), phosphoglucose isomerase (PGI), glucokinase (GK), 2-keto-3-deoxy-6-phosphogluconate (KDPG), KDPG aldolase (EDA). EDD refers to the Entner-Doudoroff pathway, and TCA stands for tricarboxylic acid.*

toglutarate (5) and allows normal growth. Glucose and gluconate metabolism are probably mainly via the Entner-Doudoroff pathway, but partly by the pentose-phosphate pathway, and the slower growth of the mutant on glucose may be related to the fructose 6-phosphate accumulated from it. Such accumulation is much higher when fructose is given, and growth on sucrose is correspondingly slower than growth on glucose.

Although the scheme qualitatively accounts for the main growth features of the mutant strain, it must be regarded as tentative, and much more mutant analysis is needed to test and elaborate it. In general, the pattern is reminiscent of pseudomonads, and in many respects the present mutant resembles a recently described *pgi* mutant in *Pseudomonas aeruginosa* (22).

The nature of the *pgi* lesion is not known, and is of some interest, particularly as several revertants show a very high level of phosphoglucose isomerase. A low level (3% or less) of phosphoglucose isomerase in the mutant would probably be sufficient for some synthesis of glucose. The small amount of exopolysaccharide in the mutant grown on arabinose had normal glucose content (studies to be reported).

The mutant should be useful in selection of various other mutational blocks in sugar pathways. It may also help to clarify the relationship between polysaccharide composition and nodulation and lead to some knowledge of which metabolic pathways are used in symbiosis.

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